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The Mycobiome, Identification and Genotyping Candida species in Clinical Isolates

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The Mycobiome, Identification and Genotyping Candida species in Clinical Isolates

A Major Qualifying Project Report submitted to the Faculty of Worcester Polytechnic
Institute in partial fulfillment of the Degree of Bachelor of Science

Submitted By:

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Submitted to:

_____ Dr. Reeta Rao

Abstract

Candida infections are common opportunistic infections among humans, especially those with an underlying immunodeficiency. Five species of Candida are most often seen clinically and these include *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, *Candida dubliniensis*, and *Candida albicans*. There is growing concern about the emergence of antimicrobial resistant and often misdiagnosed species of *Candida albicans* and *Candida dubliniensis*. This study will lay the foundation to study the mycobiome and differentiate between the species of Candida. Clinical isolates were inoculated on CHROMagar for phenotypic pre-identification after 48 h of incubation at 37 °C. Candida species were genotyped by PCR using species-specific primers for the ITS regions followed by a PCR-RFLP digestion. This method was especially important for colonies identified as *C. albicans* and *C. dubliniensis* on CHROMagar since they appear similar and are often misdiagnosed when presented clinically.

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Table of Contents

Table of Contents	4
Table of Figures	5
Table of Tables	5
Table of Supplementary Figures	5
Introduction	6
Vaginitis	8
Disease Causing Candida Species	8
Identification of Candida Species Using Differential Growth Media	9
Internal Transcribed Spacer Region	11
The Goal	12
Materials and Methods	13
Strains used in this study	13
Media and Culture Conditions	13
Control Plates	14
Control Plate of Mixed Candida	15
Serial Dilution Plates	16
Identifying using Restriction Digestion Sites in Candida Sequences	16
Streaking for a Single Colony and extracting DNA	16
Polymerase Chain Reaction of ITS region	17
Restriction Digestion	18
Genotypic identification using Engineered Restriction Enzyme Sites	19
Primer Design	19
Genomic DNA Isolation	20
Genotyping PCR	21
Restriction Digestion	21
Running on an PAGE gel	22
Results	24
Phenotypic Identification on CHROMagar	24
Using Restriction Sites natively present in all species	27
PCR-RFLP Results	29
Summary of Findings	33
Discussion	36
References	39
Appendix	41

Table of Figures

Figure 1: <i>Candida albicans</i> species under the microscope.....	7
Figure 2: The ITS region in a fungal transcription unit.....	12
Figure 3: Outline of the procedure for plating of <i>Candida</i> Species onto CHROMagar.	15
Figure 4: <i>Candida</i> spp. struck out on CHROMagar and incubated at room temperature, 30, 37, and 45 degrees.....	24
Figure 5: <i>Candida</i> spp. serially diluted and plated on CHROMagar to observe and compare growth on one plate.....	26
Figure 6: <i>Candida albicans</i> (R) and <i>Candida dubliniensis</i> (L) grown at 30 degrees. The two species are indistinguishable based on the coloration.....	27
Figure 7: Positive PCR result using previous literature primers. 100 bp Ladder (Lane 1), <i>Candida albicans</i> (Lane 2) and <i>Candida dubliniensis</i> (Lane 3)	28
Figure 8: Digestion using <i>HaeIII</i> and <i>DdeI</i> of amplified <i>C. albicans</i> and <i>C. dubliniensis</i> sequences using primers from literature. 100 bp Ladder (Lane 1), No DNA (Lane 2), uncut <i>C. albicans</i> (Lane 3), <i>C. albicans</i> cut with <i>DdeI</i> (Lane 4), uncut <i>C.</i> <i>dubliniensis</i> (Lane 5), <i>C. dubliniensis</i> cut with <i>DdeI</i> (Lane 6), 100 bp Ladder (Lane 8), No DNA (Lane 9), uncut <i>C. albicans</i> (Lane 10), <i>C. albicans</i> cut with <i>HaeIII</i> (Lane 11), uncut <i>C. dubliniensis</i> (Lane 12), <i>C. dubliniensis</i> cut with <i>HaeIII</i> (Lane 13).....	28
Figure 9: Annealing gradient to determine the annealing temperature for the <i>PacI</i> RE primer. Annealing temperatures of 40, 45,50, 55, and 60 degrees increasing left to right. (Lane 1) 100 bp ladder, (Lane 2,4,6,8,10) <i>Candida albicans</i> at all annealing temperatures and (Lane 3,5,7,9,11) <i>Candida dubliniensis</i> at all annealing temperatures.....	32
Figure 10: Decision matrix for phenotypic identification of <i>Candida</i> spp.	34
Figure 11: PCR of the ITS region and PCR-RFLP digestion methodology.	35

Table of Tables

Table 1: Phusion protocol for PCR of <i>Candida</i> species.....	17
Table 2: All primers tested throughout the development of a new method.....	29

Table of Supplementary Figures

Supplementary Figure 1: Searching for the <i>Candida albicans</i> ITS region in <i>Candida</i> Genome Database.....	41
Supplementary Figure 2: Seeing the ITS region in <i>Candida</i> Genome Database and being able to retrieve the sequence using the links below.	42
Supplementary Figure 3: Looking for homologous sequences in NCBI to find additional ITS regions in other species in addition to <i>C. albicans</i>	43
Supplementary Figure 4: Aligning ITS regions in Geneious to help identify single base differences to design a primer.	44
Supplementary Figure 5: Designing a primer in geneious.	45

Introduction

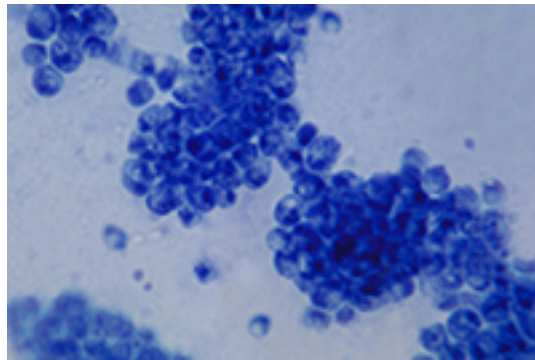
Vaginal infections, yeast infections, affect over 1 million women each year and it is uncomfortable and frustrating. Nearly 75% of women have had a yeast infection and many have had the infection greater than one time Wright (2009). The *Candida* spp. behind these infections is most often *candida albicans*. Although, there are over 200 species of candida in the world and about 20 are associated with human or animal infections Segal (2005). Candida infections are one of the most commonly discussed fungal infections and can be superficial or systemic. Systemic infections may affect vital organs since candida is carried through the blood stream throughout the body. Superficial infections typically affect cutaneous or mucocutaneous tissues such as vaginitis, thrush, or conjunctivitis.

Candida is a part of the normal microbiota or mycobiome and is present in the mouth, the gastrointestinal tract, and female vaginal tract. Under certain conditions where the host is immune-compromised or treated with broad-spectrum antibiotics, Candida may overgrow and becomes problematic. For this reason, pregnant women, those with diabetes, or immunocompromised individuals are at the greatest risk for contracting a yeast infection. Other factors such as hormonal changes, stress, and changes in acidity can increase women's chances of contracting a yeast infection ("Vaginal Candidiasis").

Differential diagnosing of a yeast infection can be difficult upon a physical exam because the symptoms are similar to many other genital infections. The symptoms of a yeast infection are itching, burning, and a characteristic thick, white

discharge. This can be in addition to pain during urination or sexual intercourse. Usually if a physician takes the time, diagnosis is done under a microscope ("The Basics of Vaginal Yeast Infections" 2015). Furthermore, identifying the etiological agent is complicated because the various *Candida* spp. Are indistinguishable from one another by microscopic observation.

Figure 1: *Candida albicans* species under the microscope.



(2015). Photomicrograph of the fungus *Candida albicans* [digital image]. Retrieved from <http://www.cdc.gov/fungal/diseases/candidiasis/>

Doctors typically prescribe a topical application such as monistat, gyne-lotrimin, and Mycelex or a single dose of oral fluconazole to treat the infection. Although, many women do not see a doctor and diagnose themselves based on the symptoms they are presenting, purchasing an over the counter treatment to fix the problem. Of these women that self diagnose and treat with over the counter medications up to 2/3 of them do not have candidiasis ("Genital/vulvovaginal candidiasis (VVC)" 2014). With the increased usage of azole over the counter medications by self-treating women, the emergence of drug-resistant *Candida* is a public health threat.

Thought to be acquired at birth through the birth canal, nursing or food, *Candida* species are part of the normal microbiota of an individual. This yeast is present in 70% of the population and kept under control by some of the body's natural mechanisms. These include immunoglobins A and G, antibodies, and polymorphonuclear leukocytes and macrophages. In addition, presence of bacterial species offer competition for limited resources thereby keeping the yeast under control. It is when the body is compromised or weakened, for example when a female is on an antibiotic prescription that kills competing bacteria, removing these controls that the species can overgrow and cause infection. These can be hospital-acquired infections and can be serious if the infection becomes blood borne.

Vaginitis

Of all the *Candida* species *Candida albicans* is found in 40% oral swabs and 20-25% of vaginal healthy swabs. A dimorphic fungus, *Candida albicans* causes 80 – 90% of vaginal yeast infections and is considered the most pathogenic of all the species of *Candida* Douglas (2002). Normally *albicans* is present as a unicellular yeast, but is seen as a budding yeast or in the hyphae form when infecting the body. This morphology change is prompted by body environment changes such as pH or temperature "Candida Albicans (Pathogenesis)". Treatment for a *C. albicans* infection is treated with prescribed fluconazole or amphotericin B.

Disease Causing Candida Species

Not all *Candida* infections are caused by *C. albicans*, about 10% to 20% of yeast infections are caused by an alternate *Candida* species "Genital/vulvovaginal candidiasis (VVC)" 2014). *C. albicans* has been considered the most pathogenic of the *Candida* species, but most recently there has been a shift decreasing the *albicans* infections and increasing alternate species infections, such as *Candida dubliniensis*. Founded and named after the city in Ireland in 1995, *C. dubliniensis* can be seen infecting patients as far back as 1950. Although *C. dubliniensis*, is often misdiagnosed as *albicans* so many infections originally thought to be caused by *Candida albicans* are diagnosed as *C. dubliniensis* retroactively. In a study done on subjects in Ireland 24.5% got a *Candida* infection from the *C. dubliniensis* species alone, but a majority had a mixed species infection with *C. albicans* being one of the species also present Donnelly SM1 (1999). *Candida dubliniensis* is phenotypically very similar to *Candida albicans* and therefore is hard to discriminate between even in testing methods such as PCR, growth, and plating. The differences in response to medications have yet to be determined, but it has been thought to confer resistance to fluconazole one of the main treatments of *Candida albicans* infections (Kenneth Oliveira, 2001). This poses a problem in treatment if misdiagnosed as *C. albicans*. The emergence of this species with little known data, in combination with the threat of misdiagnoses, calls for a more advanced method of detection.

Identification of *Candida* Species Using Differential Growth Media

A simple way of identifying different species is phenotypically on some type of chromogenic medium. Chromogenic medium is a type of media that allows for differentiation of organisms based upon the color they present. Some chromogenic media that researchers are familiar with are ONPG, X-gal, and X-glu. Chromogenic media is composed of chromogens, which are then further made up of a substrate and a chromophore. The substrate is designed to target a specific enzyme activity of the organism. When the enzyme is released it cleaves the substrate, cutting the chromogen in half, releasing the chromophore. These unconjugated chromophores express phenotypically as different color precipitates to the eye of an observer (Siegrist, 2016). Depending on the color observed, the species/organism could be discovered. The reason this type of media is so popular is it's rapid identification and ease. In comparison to other experiments that might be used to discern between species, this result will come in a shorter time. There are no special instruments or understanding needed to use this type of media and one type can delineate between multiple organisms. This type of media has a wide range of use in clinical biology, industrial microbiology, food and beverage quality control, and water testing. The first type of chromogenic media was made to identify E.coli types. Some notable uses now are for MRSA/ORSA and Difficile infections, Listeria, and *staphylococcus aureus* ("Chromogenic Media,") . In the identification of Candida the brand of chromogenic media, CHROMagar is used. CHROMagar is made for multiple identifications in clinical microbiology, but the one made for Candida differentiates after 36-48 hours between *Candida albicans* appearing green, *Candida krusei* appearing metallic blue, *Candida tropicalis* appearing fuzzy pink, and other

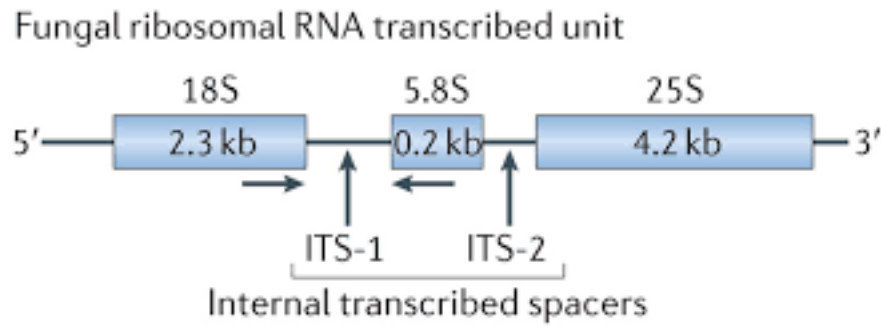
species appearing white. Occasionally though species can't be differentiated between, such as *Candida albicans* and *Candida dubliniensis*, two species that are often mistaken for one another. *Candida dubliniensis* is expected to appear a dark green while *Candida albicans* should appear a blue green. Although, according to previous literature research unless the *C. dubliniensis* sample is observed from primary plating this will not be easy to see as the colonies can undergo phenotypic switching. In that case *C. dubliniensis* and *C. albicans* are difficult to differentiate and a misdiagnoses could result in the inability to treat the infection. For this reason a genomic identification is necessary.

Internal Transcribed Spacer Region

While CHROMagar can ensure that the genus is *Candida*, a more genomic look at the yeast can provide species and subspecies identification. This can be done through observation of the ITS, or internal transcribed spacer regions. ITS regions are variable stretches of DNA that allow for identification of species and subspecies. The rRNA genes around this region are highly conserved meaning that between species they do not show much variability and can only be used to identify on a taxonomic level the sample. The ITS region spans from the 18S to the 25S regions and is further subdivided into ITS1 and ITS2. ITS 1 covers 18S until 5.8S while ITS 2 stretches from 5.8S until 25S (Christian Pitulle, 2002). Due to their lack of conservation these regions have great variability between them and little homology between species of yeast, differing in size and sequence. This is what makes these

regions the perfect targets for molecular analysis. Although there is no established database of the sequences so it is difficult to find ITS sequence information for different yeasts.

Figure 2: The ITS region in a fungal transcription unit.



(2014). Fungal RNA transcribed unit [digital image]. Retrieved from http://www.nature.com/nri/journal/v14/n6/box/nri3684_BX1.html

The Goal

The goal of this major qualifying project was to develop a clear, easy method to identify and differentiate between the *Candida* species, specifically *Candida albicans* and *Candida dubliniensis*. To do this CHROMagar was used to phenotypically identify the species as either *C. albicans* or *C. dubliniensis*. Once selected as one of the species in question a colony was extracted and the ITS region was amplified using PCR. To genotype, primers used in the PCR contained a single base difference for *candida dubliniensis* that when amplified through a polymerase chain reaction with a RE site surrounding the single base difference would not allow the DNA to be cut if it were *C. dubliniensis*. The resulting DNA fragments were separated by gel electrophoresis, revealing two bands if the restriction site was present, else a single, larger fragment for the undigested PCR product.

Materials and Methods

The research procedures that were used throughout the project are summarized in this section with all necessary additional details in the appendix.

Strains used in this study

The five reference strains of different *Candida* species used were: *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. krusei*, *C. glabrata*.

Media and Culture Conditions

The main media that was used during the research was CHROMagar. CHROMagar can be made at 47.7 grams per liter, although in research only smaller amounts such as 200 mL volumes were plated at a time because the plates only last a week being stored at 4 degrees Celsius. So more frequent media preparation in smaller amounts is advisable. A flask is used and the weighed material, 9.54 grams if making a final volume of 200mL, is added to the empty flask. A graduated cylinder allows for accurate volume addition of water into the media powder. Stir the media and water mix gently by swirling the flask, to partially homogenize the mixture before covering the top of the flask with saran wrap and placing it into the microwave to be heated. The amount of media is so small that after about 30 seconds in the microwave the solution will need to be taken out and stirred before replacing the flask in the microwave for a final 30 seconds to 1 minute. Once the

solution starts bubbling in the microwave it should be taken out and stirred gently just with a gloved hand. If the solution is left in longer than the bubbling point it will only take seconds for it to boil over. The solution at the boiling point should be clear and the solution should be a homogenous mixture with no visible agarose powder. The media at this point can sit on the lab bench covered by the saran wrap to cool while the hood is sterilized with ethanol and prepared for plate pouring. Once the media has cooled for about 10 minutes, or is able to be handled without a heat glove, the plates are poured or pipetted at 20 mL per plate. After roughly one hour the plates should be hardened enough for use. Before using it is important to check that the surface of the plate is not wet to ensure an uncompromised plating of samples. Both control plates of all reference strains and a control plate of mixed colonies were plated to gain a reference point for future experiments as to the phenotypic expression.

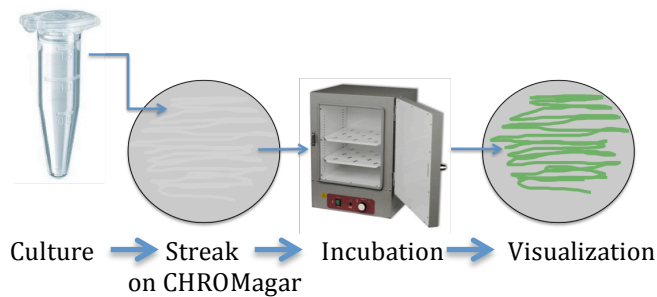
Plates that are not used or are taken out of the incubator and are going to be stored for later use are wrapped in aluminum foil as the plates are light sensitive and stored at 4 degree upside down to prevent condensation build up on the surface of the plate.

Control Plates

Control plates were made from reference strains in the freezer. After taking the reference strains out of the -80-degree freezer they were put on ice immediately. Then, an autoclaved wooden spreading stick was dipped into the reference strain, and whatever sample was on the stick was spread onto the plate. This should all be done by an open flame to limit contamination and should be done very quickly to

prevent thawing of the reference strain. If all reference strains are being plated, doing two types at a time is probably best rather than taking the time to spread 6 strains and leaving the samples out of the -80 that long. The plates are then put in the 30-degree incubator for 24 hours and observed the next day for growth.

Figure 3: Outline of the procedure for plating of Candida Species onto CHROMagar.



Control Plate of Mixed Candida

While control plates of single species candida are important to determine how single species will be identified, samples of mixed Candida species were used as well as some infections might present as multiple species and it is important to visualize what that would look like. Although, the mixed plates could not be done in the same way as single species plates because the colonies needed to be dilute to prevent overgrowth of one species on the plate covering the others. To make a mixed control plate, 500 microliters of YPD were put into a micro centrifuge tube and one colony from each single species candida plate was put into the YPD. The mixture was vortexed and then a toothpick worth of mixture was struck out onto a CHROMagar plate in an A-C streak method. The plates were then put in the 30-degree incubator for 24 hours and observed the next day for growth.

Serial Dilution Plates

To observe growth and colors at less concentrated sample amounts the samples were serially diluted and plated. All species of *Candida* were grown in YPD overnight. The absorbance of the sample was then observed using the spectrophotometer. All samples were then diluted to an absorbance of 1. Then five 10 microliter serial dilutions were done in 1.5 mL conical tubes. For each species each of the five dilutions were plated in 10 microliter drops on the CHROMagar plate. In this way all dilution amounts and all species could grow on the same plate further helping compare species phenotypes.

Identifying using Restriction Digestion Sites in *Candida* Sequences

To start the process of genomic ID of *Candida* species the first methods were based off of a proven protocol from the literature review (J Irobi, 1999). In this way the results could help form a new protocol.

Streaking for a Single Colony and extracting DNA

To get the DNA necessary for future PCR a single colony is struck out and used. Using the A-C streak method a single colony is struck out for on CHROMagar and after 24-48 hours the plates can be checked for single colonies. If the colonies are not well defined as single colonies a restreak from the original plate onto a new plate will help define them further. One single colony from the plate is used for DNA extraction and placed into a microcentrifuge tube with thirty microliters of 0.2%

SDS. The microcentrifuge tube is vortexed for 15 seconds and then put in a 90-degree heat block for 4 minutes. Lastly, the tube is microcentrifuged for 60 seconds at 14.5 rpm, and then the supernatant is transferred to a brand new 1.5 mL microcentrifuge tube. This DNA will not last long in -20 storage as it is dirty DNA from the quick colony protocol.

Polymerase Chain Reaction of ITS region

DNA was prepared from various *Candida* spp. and primers specific to the ITS region were designed as previously described (J Irobi, 1999). The primers used were 5'-GGAAGTAAAAGTCGTAACAAGG-3' and 5'-GGTCCGTGTTTCAAGACGG-3'.

The protocol for PCR preparation is to add the following to a PCR tube to get a final volume of 20 µl:

- 10 µl 2x Phusion Buffer Master Mix
- .4 µl each primer at a concentration of 25 µM
- .5 µl DNA template
- 9.1 µl DI water

The PCR was then run through the phusion PCR protocol that was:

Table 1: Phusion protocol for PCR of *Candida* species.

1. 98 degrees for 30 seconds
2. 98 degrees for 5 seconds
3. 50 degrees for 10 seconds
4. 72 degrees for 2 minutes

5. Steps 2-4 are repeated for 30 additional cycles
6. 72 degrees for 5 minutes
7. The sample is held at 4 degrees for infinite time

To check that the PCR worked a gel is run through gel electrophoresis. The gel is made as a 2% gel by putting 1.5 grams of agarose into 75 mL of TAE buffer. This mixture is then heated to liquefy in the microwave for about 1 minute. In its liquid form 6 microliters of ethidium bromide are added and dissolved into the mixture. Once solidified the gel reservoir is filled with TBE buffer and the sample is loaded into it's respective wells. The gel is then run at 80 mV for about 2 hours.

Restriction Digestion

If the gel shows a positive PCR result, the sample is digested. The sample can be digested with one of three enzymes; BfaI, HaeIII, and Ddel. Restriction enzymes were chosen with help from previous literature (J Irobi, 1999) In digestion 1 µg of DNA is used. Comparing the band size against the ladder size can determine about how much DNA is present in the sample. A nanodrop cannot be used because the sample has not been cleaned up from the PCR or SDS. In addition to the DNA, Restriction enzyme, cut smart 10x buffer, and DI water are added to a 1.5 mL conical tube to incubate overnight at 37 degrees celsius. After an overnight digestion the sample is run on a gel. A 2 percent gel is made and run at 80 mV for about 2.5 hours. The gel is run as close to the end as possible to get the best separation between bands. The samples are run against a 100 bp ladder.

Genotypic identification using Engineered Restriction Enzyme Sites

The literature results seemed to show differentiation, but the restriction enzymes were rare and the bands were tricky to separate enough to observe the differences between species. Therefore, a method was designed to use a more common restriction enzyme and have a simpler result shown.

Primer Design

The region chosen to amplify and observe differences between was the ITS1 and ITS2 region of *Candida albicans* and *Candida dubliniensis*. The first step was to identify the sequences of the regions in these particular species. To do this the *Candida albicans* sequence was discovered on Candida Genome Database. Using the gene/sequence resources of the database, the ITS gene was searched for under the Candida Assembly 22 with base pairs upstream and downstream to incorporate the ITS 1 and ITS 2 regions.

Once submitted, this allows for observation of the area the sequence is coming from and to be able to retrieve the sequence using the FASTA link.

It was thought that the other species would be found through candida genome database as well, but when searching for those sequences CGD returned no sequence found. So, NCBI had to be used to determine the other sequences, the most relevant for this project being *Candida dubliniensis*. The *Candida albicans* sequence that was found was copied and blasted in to NCBI to look for homologous

regions in other species. To do this the sequence is plugged into the BLAST portal and the search button is pressed to bring the user to the homologous sequences web page.

With the sequences found the primers could be created. Only primers were created for amplification of *Candida albicans* and *Candida dubliniensis*. This was to simplify the process and because these two species had the most phenotypically similar expression. The software Geneious was used to align *Candida albicans* and *Candida dubliniensis*. Wild type control primers were made first with bases that match in both *C. albicans* and *C. dubliniensis*. Next a single mismatched base to make primers around was found.

The single base mismatch was sought for on the 5' end and 3' end because it was uncertain which would work in the experiment and a wild type primer was made on each end about 500 base pairs upstream from the respective base mismatch primer. The primers created were between 18 and 24 bases in length and had a GC content less than or equal to 50%. The sequence for *C. albicans* was used as the primer even with the single base pair difference between *C. albicans* and *C. dubliniensis*. This would make the SNP primer.

With the single base mismatch identified a restriction enzyme cut site was made around the mismatch to cut at the mismatch for *C. albicans* and not *C. dubliniensis*.

Genomic DNA Isolation

To test the primer function and ensure the protocol is working genomic DNA is used rather than colony PCR, to avoid any problems in the experiment being caused by poor DNA. The genomic DNA is extracted using the Qiagen Yeast Star genomic DNA kit which can use a colony or grown DNA culture. In this case one colony was grown overnight on a rotator wheel in 3mL of YPD media. Then following the steps for Protocol 2 in the kit, the genomic DNA is extracted. Once the DNA is extracted it is nanodropped to get an accurate concentration for further experiments. One thing to note when using the nanodrop is whether the concentration reading shows an ethanol peak or is reading pure DNA. If the concentration is extremely high the nanodrop may be reading left over ethanol from the elution and the DNA is not completely pure, this should be avoided.

Genotyping PCR

The PCR's that were run were done with WT primers, the SNP primers, and RE primers. All PCR's were run under the "yeast col" program. To establish annealing temperatures, gradient PCR's were run for each primer reaction. Annealing temperature of WT reactions was 63 degrees and the restriction enzyme digestion annealing temperatures varied.

Restriction Digestion

After a positive RE PCR the samples were digested with the restriction enzyme. The digestion was run with 1ng worth of DNA, restriction enzyme, and 10x Cut smart Buffer that is compatible with the restriction enzyme. The total volume of the reaction is dependent on how much DNA is needed to make 1 ng. The digestion is run for 1 hour at 37 degrees Celsius in the thermocycler. A water bath could also be used, but the thermocycler was found to hold the temperature stable more reliably.

Running on an PAGE gel

Making the primer region, the DNA region to be amplified, smaller helped when trying to see whether digestion occurred. A change in band size from 200 to 180 base pairs is slightly more distinguishable than a change from 500 to 485. Although, with either the separation is important to be able to clearly see digestion. This cannot be seen through running DNA on even a .8% agarose gel and has to be done by running on a poly acrylamide gel. To set up an SDS page gel the appropriate volumes of acrilimide-bis acrilimide, water, tris buffer, APS, and TMED are put in a conical tube for a total of 10mL. Once the APS and TMED are added the solution must be moved quickly to the gel chamber because it will harden quickly, therefore those should be added last and quickly. Once the gel solidifies in the chamber set-up it can be loaded with 10 microliters of each sample. The uncut and ladder samples are prepared with 5 microliters of uncut or ladder sample, 5 microliters of water and 2 microliters of gel loading dye. The cut samples are prepared with 10

microliters of sample and 2 microliters of loading dye. The gel is run at 150-200 volts dependent on the gel percentage. Most often 10% was used and run at 200 volts. The sample is run until the loading dye moves far enough down the gel to signify the bands have separated enough.

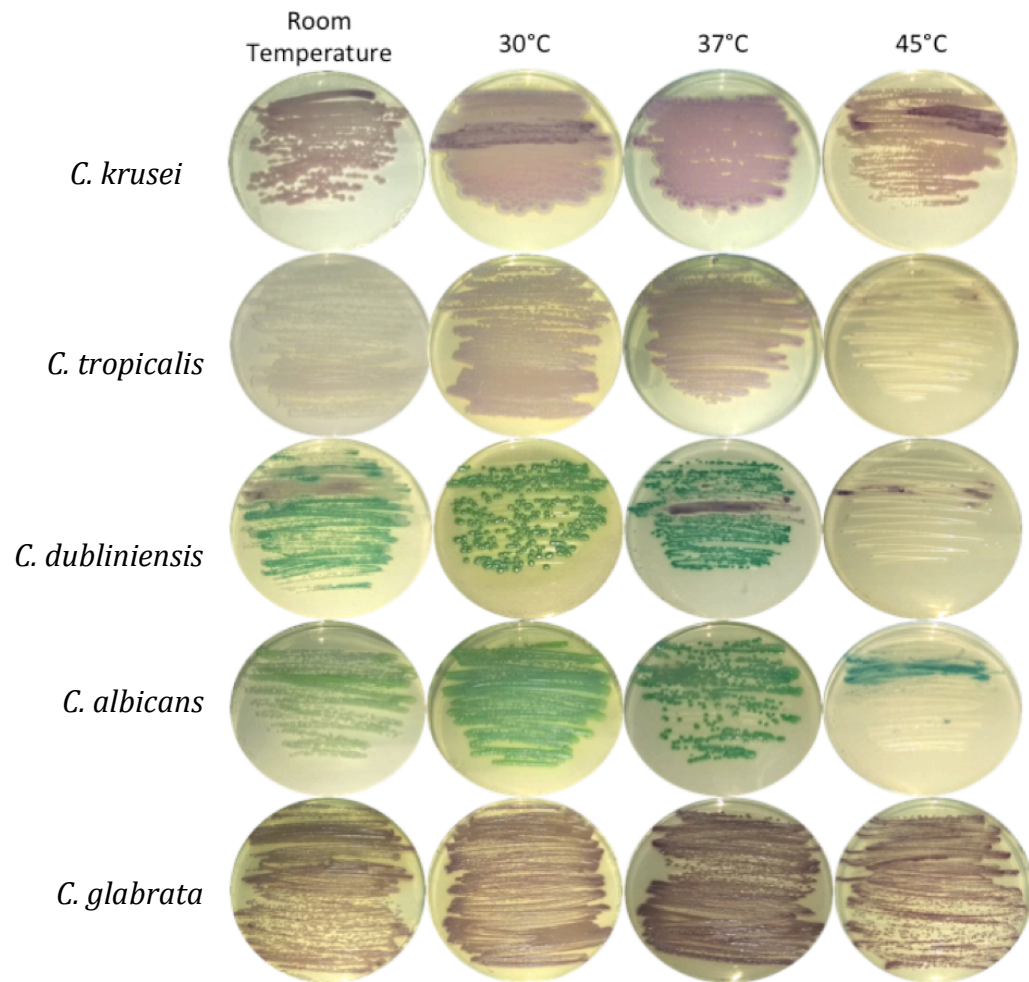
Results

The goal of this project was to develop an easy, rapid test method to identify species of *Candida* that could be causing infection in patients, specifically *Candida albicans* and *Candida dubliniensis*.

Phenotypic Identification on CHROMagar

Phenotypically *Candida* spp. can be differentiated through their growth on CHROMagar plates. Dependent on the chromophore the species is known to release, a color is expressed such as hues of green, brown, purple, or pink.. In Figure 4 the plates of five species of *Candida* can be seen after growth for 48 hours at room temperature, 30 degrees, 37 degrees and 45 degrees. These species are *Candida albicans*, *Candida dubliniensis*, *Candida krusei*, *Candida tropicalis*, and *Candida glabrata*.

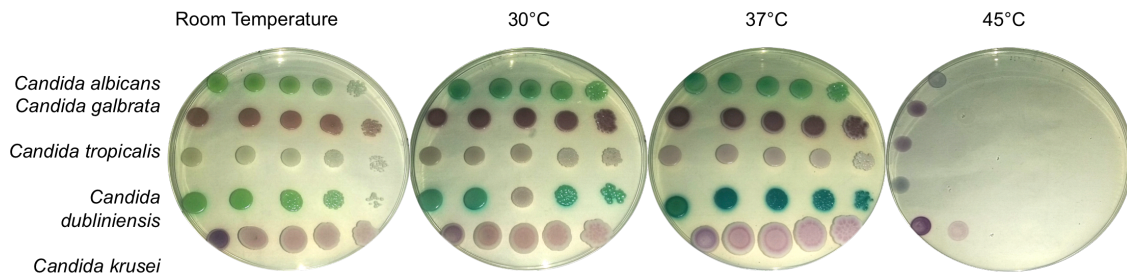
Figure 4: *Candida* spp. struck out on CHROMagar and incubated at room temperature, 30, 37, and 45 degrees.



The CHROMagar plates show that almost all of the species present as a different identifying color when struck on CHROMagar. *Candida tropicalis* grows a light shade of brown and can be easily differentiated from *Candida glabrata*, which is a dark mahogany brown. *Candida krusei* is a unique light pink or light purple color. Finally, the CHROMagar plates show that both *Candida albicans* and *Candida dubliniensis* appear as the same shade of green at almost every temperature except 45°C. At this temperature they appear as a purple streak for *C. dubliniensis* and a green streak for *C. albicans*.

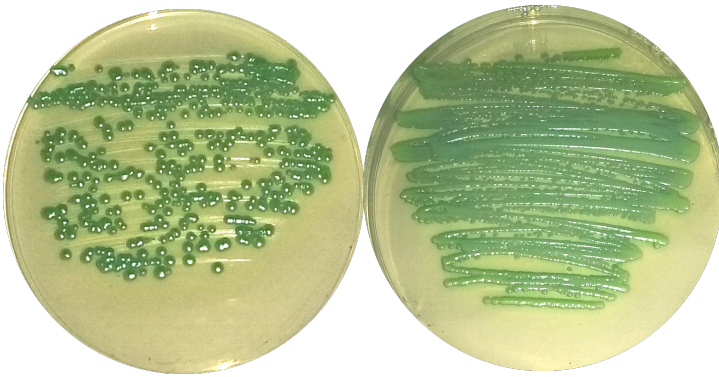
Serial dilutions of cultures of each species were spotted on CHROMagar. This allowed for closer comparisons of color and to observe the amount of growth for each species over the course of 48 hours. This can be seen in Figure 5.

Figure 5: *Candida* spp. serially diluted and plated on CHROMagar to observe and compare growth on one plate.



The dilution plates show that *Candida krusei* always has larger growth compared to the other species, which is best seen at 45°C where *C. krusei* shows more growth than any of the other strains. *C. krusei* also seems to present with undefined edges showing more abnormal circular growth in comparison to other species. *C. dubliniensis*, *C. albicans* and *C. tropicalis* can be seen having less growth in the smallest dilution series at room temperature, but not at any other temperature. *C. glabrata* shows almost identical growth at every temperature, but 45°C. Also, at the lowest dilutions, or the farthest right droplet, growth has undefined edges and does not stay circular in form. Even with serial dilutions it is hard to see phenotypic differences between *C. albicans* and *C. dubliniensis*. This is best seen when the respective plates are seen side by side after incubation at 30 degrees for 48 hours. This can be viewed in Figure 6 below.

Figure 6: *Candida albicans* (R) and *Candida dubliniensis* (L) grown at 30 degrees. The two species are indistinguishable based on the coloration.

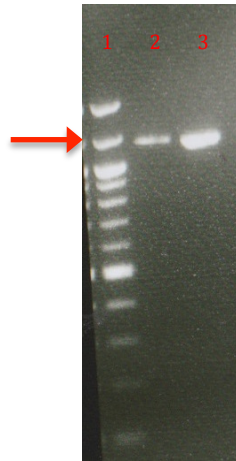


These strains are almost visually indistinguishable. The CHROMagar plates show that there can be phenotypic identification for many of the species, but for some, such as *Candida albicans* and *Candida dubliniensis* phenotyping is not clear enough. To provide support for the phenotypic results genotypic identification can be done using a combination of polymerase chain reactions, restriction enzyme digestion, and gel electrophoresis DNA separation. This type of experiment had been done in previous literature. The first set of results that were achieved were in an attempt to reproduce those already found in literature and gain an understanding of identifying *Candida* spp. genotypically.

Using Restriction Sites natively present in all species

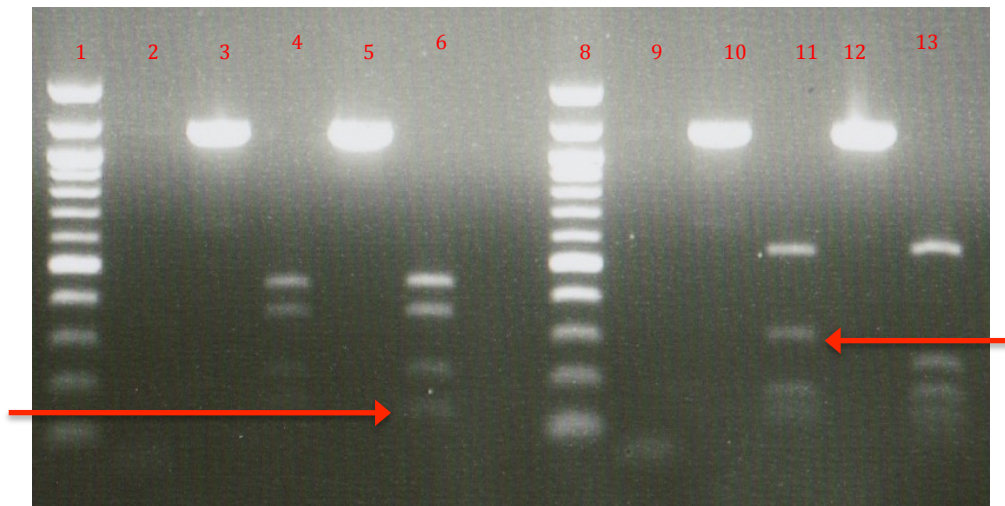
A PCR was run with primer sequences from the literature (J Irobi, 1999) and gel electrophoresis was done to show that the DNA could successfully go through a polymerase chain reaction with those primers.

Figure 7: Positive PCR result using previous literature primers. 100 bp Ladder (Lane 1), *Candida albicans* (Lane 2) and *Candida dubliniensis* (Lane 3)



With a positive PCR result the multiplied DNA could be cut using restriction enzymes *HaeIII* and *DdeI* at sites present in all candida species sequences.

Figure 8: Digestion using *HaeIII* and *DdeI* of amplified *C. albicans* and *C. dubliniensis* sequences using primers from literature. 100 bp Ladder (Lane 1), No DNA (Lane 2), uncut *C. albicans* (Lane 3), *C. albicans* cut with *DdeI* (Lane 4), uncut *C. dubliniensis* (Lane 5), *C. dubliniensis* cut with *DdeI* (Lane 6), 100 bp Ladder (Lane 8), No DNA (Lane 9), uncut *C. albicans* (Lane 10), *C. albicans* cut with *HaeIII* (Lane 11), uncut *C. dubliniensis* (Lane 12), *C. dubliniensis* cut with *HaeIII* (Lane 13).



The separation of the DNA bands through gel electrophoresis shows that *C. albicans* and *C. dubliniensis* can be identified as different species through slight differentiations in the digestions.

PCR-RFLP Results

A new method was developed to conclusively distinguish between the *Candida albicans* and *Candida dubliniensis* species using the highly divergent ITS1 and 2 regions. The intent of the method was to engineer a restriction site around a single base pair difference in *Candida albicans* and *Candida dubliniensis*. Several primers were developed to understand how to amplify the restriction site that was inserted and successfully cut in solely *C. albicans* and not *C. dubliniensis*.

Table 2: All primers tested throughout the development of a new method.

<u>Primer Name and Sequence</u>	<u>Description</u>	<u>Did it work?</u>
WT 3': 5' CCTGATTTGAGGTCAAAGTTTGAAG 3'	Made to check whether the primer set up was correct and where a positive PCR band would lie	Yes
WT 5': 5' CACCGCCAAAGCAAGTTTG 3'	Made to check whether the primer set up was correct and where a positive PCR band would lie	Yes
3'SNP: 5' GTTAGACCTAAGCCATTGTCAAAGC 3'	Control to show positive PCR with a SNP between <i>C. albicans</i> and <i>C. dubliniensis</i>	No
5' SNP: 5' CCACCGCCAAAGCAAGTTTGTTC 3'	Control to show positive	No

	PCR with a SNP between <i>C. albicans</i> and <i>C. dubliniensis</i>	
5' <i>Eco</i> RI site inserted: 5' CCACCGCCAAAGCAAGTT GAATTC 3'	First attempt at placing a restriction site in at the end of the primer with the SNP included in the primer sequence	No
3' <i>Eco</i> RI site inserted: 5' GTTAG AATTC AGCCATTGTCAAAGC 3'	First attempt at placing a restriction site in at the end of the primer with the SNP included in the primer sequence	No, couldn't seem to get a positive PCR result with a 3' mutated primer
5'_SNP_ <i>Xba</i> I - 5' CAGAGG T CATAAACTTACAACC 3'	Trying another SNP and a different placement in the primer	Yes
5'_RE_ <i>Xba</i> I - 5' CAGAGG TCTAG AACTTACAACC 3'	Different placement in the primer	Yes amplified, but did not see a cut, realized the cut is only 7 base pairs in a total amplification of 400 so it can't be seen easily
5'_SNP_ <i>Hind</i> III - 5' GGCCCC AG CTGCCGCCAGA 3'	Trying another SNP and a different placement in the primer	Yes
5'_RE_ <i>Hind</i> III - 5' GGCCCC AAGCTT CCGCCAGA 3'	Different placement in the primer	Yes amplified, but did not see a cut, realized the cut is only 7 base pairs in a total amplification of 400 so

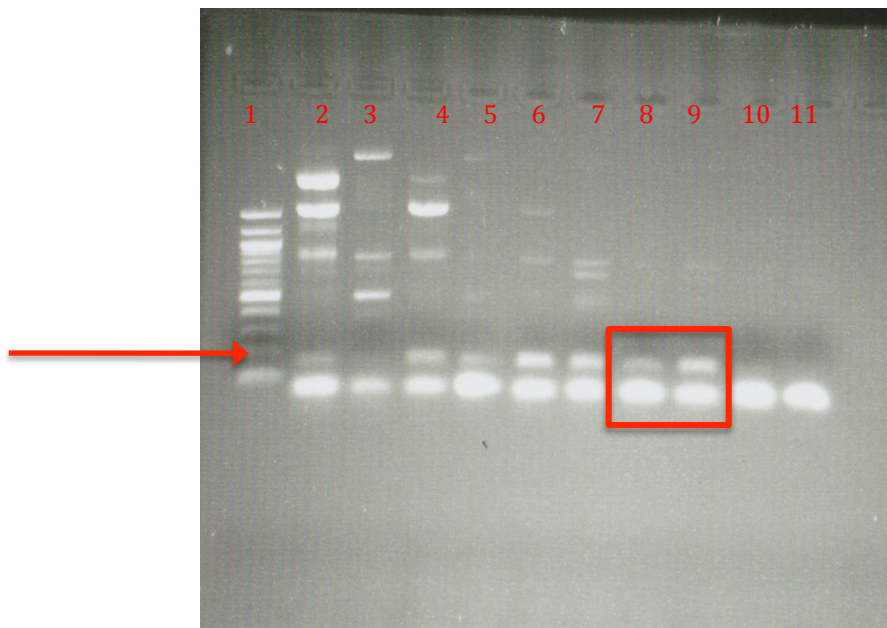
		it can't be seen on agarose
5'_RE2_HindIII 5' AACAAACTTGCTTTGGCGGTGGGCCCC AAGCTT CCGCCAGA 3'	Extending the primer length to allow for a larger digestion so the digest is visible	Yes this amplified, but both samples were cut (<i>C. albicans</i> and <i>C. dubliniensis</i>)
3'_WT_HindIII 5' GAATATCTGCAATTCATATTA 3'	Moving the WT primer closer so the total amplification region is smaller	Yes
5'RE_ScaI: 5' TAAACTTACAACCAAATTT GAGCT 3'	Not placing the SNP in the primer, but allowing it to complete the RE site	No, no amplification seen
5'RE_PacI: 5' AAACCTTGCTTTGGCGGT TTAATTA 3'	Not placing the SNP in the primer but allowing it to complete the RE site, a different location than SacI	Yes amplification seen and appropriate digestion

All primers were stored in the minus 20 fridge next to the lab bench in a box labeled Heather Bartlett's Primers. The most successful of these primers was the 5'RE_PacI primer.

The polymerase chain reaction was first tested with the wild type primer and those containing the single base pair mutation. In this way it could be ascertained that the primers were made correctly and provided a control PCR that would show a positively amplified band to compare mutated primers to.

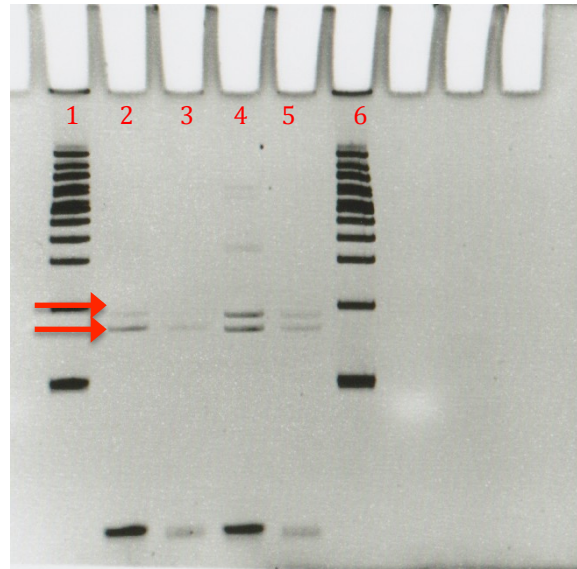
With it established where a positive PCR band lies when run through a gel electrophoresis apparatus, the restriction enzyme primer was used in PCR amplification. This result was then separated through gel electrophoresis to determine whether the amplification was successful even with the inserted base pairs to make a restriction site.

Figure 9: Annealing gradient to determine the annealing temperature for the *PacI* RE primer. Annealing temperatures of 40, 45, 50, 55, and 60 degrees increasing left to right. (Lane 1) 100 bp ladder, (Lane 2,4,6,8,10) *Candida albicans* at all annealing temperatures and (Lane 3,5,7,9,11) *Candida dubliniensis* at all annealing temperatures.



The band is expected to be 184 bp and the PCR fragment appears without any non-specific bands in an agarose gel at around 200 bp when annealed at 60 degrees, which clarifies that this is the band that is desired. Therefore, the DNA can be digested with the *PacI* restriction enzyme to discriminate between *C. albicans* and *C. dubliniensis*.

Figure 10: *Candida albicans* and *Candida dubliniensis* digested with *PacI*. (Lane 1+6) 100 bp Ladder, (Lane 2) uncut *C. albicans*, (Lane 3) cut *C. albicans*, (Lane 4) uncut *C. dubliniensis*, (Lane 5) cut *C. dubliniensis*.

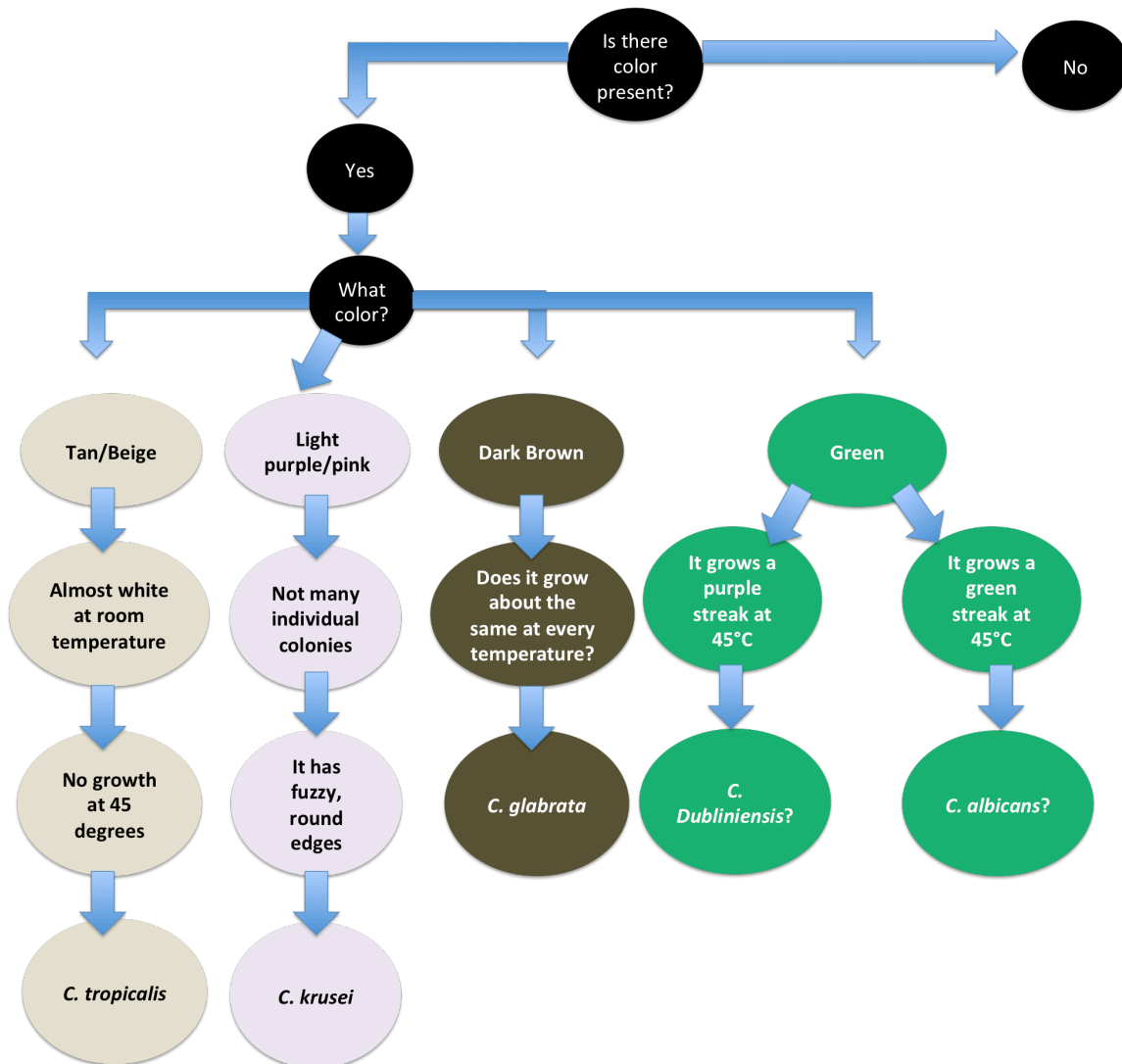


Running on a page gel shows that an uncut amplified DNA has two bands while the cut *C. albicans* has a single band. Uncut *C. dubliniensis* in lane 4 and cut *C. dubliniensis* in lane 5 of figure 10 appear with two bands. This delineates between *C. albicans* and *C. dubliniensis*.

Summary of Findings

Clinical isolates can be obtained and struck out on CHROMagar for phenotypic identification. All Candida species have a unique morphology and color when seen on CHROMagar. The table below shows a process method to identifying Candida species on CHROMagar.

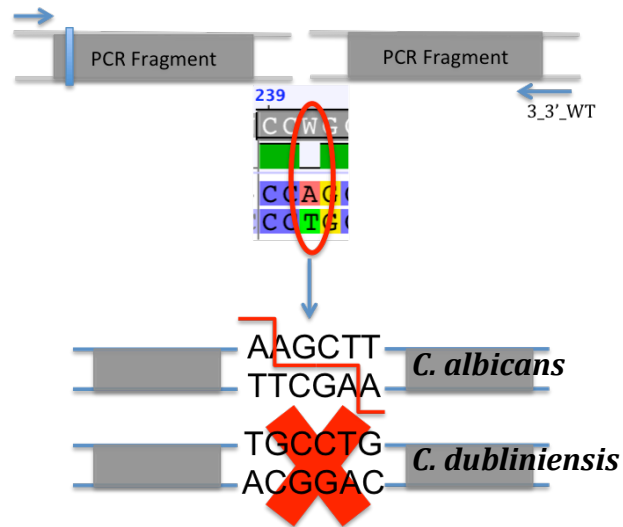
Figure 10: Decision matrix for phenotypic identification of *Candida* spp.



As can be seen, there is no convincing phenotype to differentiate between *C. albicans* and *C. dubliniensis* besides a single streak at 45°C. For these specific species genotypic identification, through amplification of the non-conserved ITS region, can more definitively argue the infecting species type. In the PCR of the ITS region the 5'_RE_PacI primer is developed to amplify the region before a single base mismatch in *C. albicans* and *C. dubliniensis*, while including that mismatch as the last base to an

engineered restriction site at the end of the primer. The figure below shows the process summarized.

Figure 11: PCR of the ITS region and PCR-RFLP digestion methodology.



Then, as seen represented by an X in Figure 11, only *C. albicans* is cut by *PaclI* because its mismatch completes the restriction site while *C. dubliniensis* does not. This appears on a 1% agarose gel as seen in Figure 9 and can more definitively tell the clinician what species is infecting the patient.

Discussion

Differentiation between candida species could play an important part in helping to diagnose and treat yeast infections in women.

Phenotypic differentiation can be done on CHROMagar for five of the candida species most seen in candida infections whether they are usually seen in blood borne, thrush, or vaginitis infections. These five species are *Candida albicans*, *Candida krusei*, *Candida dubliniensis*, *Candida glabrata*, and *Candida tropicalis*. All of these species present as a unique color except for *Candida albicans* and *Candida dubliniensis*. These two species present as green colonies on CHROMagar and even the same shade of green at all temperatures except 45 degrees. At 45 degrees *Candida albicans* has a sole green streak that grows, while *Candida dubliniensis* has a sole purple streak that appears after 48 hours. Only this plate could really give the experimenter an inkling as to which species is present in the patient. The results are not enough to say what species is affecting the patient and treat them.

Genotypic identification is an additional test to accompany the phenotypic results and more confidently claim which species is present. Previous literature establishes identification through digestion of the sequence using a native restriction site in all five species and sequences. The experiment is designed to digest all five-reference sequences although it was only used for *Candida albicans* and *Candida dubliniensis* in this experiment because of the misdiagnoses often occurring due to phenotypic similarity. As can be seen in figure 8 the digestion of *Candida albicans* and *Candida dubliniensis* using this method, with either *DdeI* or *HaeIII* restriction enzyme, shows small differences between the two strains and

must be run carefully to get the correct amount of separation on agarose to see the differences in the bands between the two species. This isn't exactly an obvious differentiation between the species and takes considerable time to run at a low voltage on a low percentage agarose gel to separate the bands and visualize the differences.

Through the identification of a single base pair difference between species of *Candida albicans* and *Candida dubliniensis*, and the development of a restriction site around the SNP, a simple method of identification could be created. Positioning the SNP at the end of the primer but not inside the primer and allowing it to complete the desired inserted restriction site allows for amplification of the DNA with the included restriction site, but while keeping the SNP in tact and not corrected. If the site and the SNP are placed inside the primer the amplification may eventually overwrite the SNP and correct it to match the primer exactly therefore both sequences will cut and differentiation would not be possible.

The *PacI* site is TTAATTAA with the last A not being in the primer and mismatching with the T present at that site in *C. dubliniensis*. Original amplification of the DNA shows only one band on an agarose gel, but when run on an SDS page gel shows two bands. When cut with the *PacI* restriction enzyme only *C. albicans* cuts, which show that amplification of the restriction site was successful and the process can provide a way of discriminating between species. Although, the amplification of the site would be better should it only amplify one band and that way cutting the site would appear much more obvious rather than going from two bands to one and not changing size. In the future the development of a primer with greater

complementarity between the desired restriction enzyme site and the native sequence could allow for more specific amplification resulting in only one band and greater visibility of a successful digestion.

The development of this procedure could provide a method to more easily identify candida species and aide in the treatment of women with frustrating infections that are often treated as *C. albicans* without a second glance. With the rise of non-ablicans species such as *C. dubliniensis* and resistance to current treatment, diagnostic methods like these will be needed.


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Appendix

Supplementary Figure 1: Searching for the *Candida albicans* ITS region in Candida Genome Database.



Candida
Genome Database

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Gene/Sequence Resources

This resource allows you to retrieve sequences and analyze them using several sequence analysis tools. Select a sequence(s) by using one of the options on this page:

1. enter a gene or open reading frame name,
- 2a. specify a chromosomal region, or 2b. upload a list of chromosomal regions,
3. enter any raw DNA or protein sequence.

After submitting the form, you will be presented with options to download and/or analyze the sequences.

Note that this resource allows you to retrieve sequences using a single gene name, or using chromosomal or contig coordinates. To retrieve sequences and other information for multiple genes using a list of standard or systematic names (e.g., ACT1 or orf19.2203), please go to the [Batch Download](#) page.

1. Enter a Name
or the first few characters followed by *:

and Select:

Examples:
Gene - ACT1
ORF - orf19.2203
CGDID - CAL0001571

If available, add flanking basepairs
upstream and downstream
☐ Use the reverse complement

OR

2a. Pick a chromosome (or contig):

Then enter coordinates (optional):
 to
☐ Use the reverse complement

The first 100,000 basepairs of the chromosome or contig sequence will be retrieved if no coordinates are entered.

OR


2b. Upload a file of chromosome (or contig) regions to download genomic sequences in batch:
 No file chosen
☐ Use the reverse complement

Chromosome regions should be specified with the following tab or space separated columns:
(i) chromosome/contig, (ii) start_coordinate, (iii) stop_coordinate
The file should contain regions from a single genomic assembly (19, 20, or 21). Entering coordinates (in columns ii and iii) is optional. The first 100,000 basepairs of the chromosome or contig sequence will be retrieved if no coordinates are entered.
C. albicans SC5314 Assembly 21 example:
Ca21chr3_C_albicans_SC5314 1356 20455
Ca21chr4_C_albicans_SC5314 11331 18001
Ca21chr6_C_albicans_SC5314 9856 100010
C. albicans SC5314 Assembly 19 example:

OR

3. Type or Paste a
 Sequence:

The sequence **MUST** be provided in **RAW format**, no comments (numbers are okay).
☐ Use the reverse complement



Candida

Genome Database

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Gene/Sequence Resources

?

The currently selected gene/sequence is :

ITS1/CR_08780W_A (ITS1) on Ca22chrRA_C_albicans_SC5314 from coordinates 1893023 to 1893161 plus 200 basepair(s) of upstream sequence and 400 basepair(s) of downstream sequence.

[\[Change Entered Selection or Coordinates\]](#)



Biology/Literature

[Locus Info](#)
[Literature Summaries](#)

Display Maps/Tables

[GBrowse](#)
[Flanking Features via BatchDownload](#)

(Features within 200 bp upstream and 400 bp downstream of the query feature)

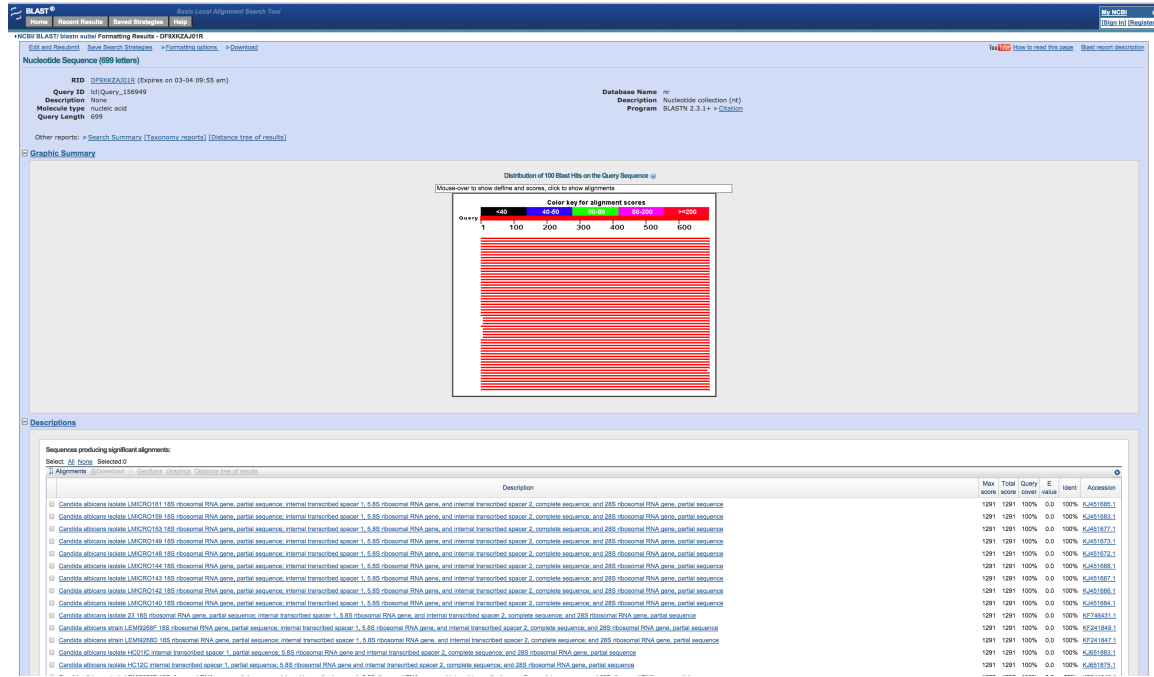
Sequence Analysis

[BLAST Search](#)
[Genome Restriction Map](#)

[Design Primers](#)

Sequence Retrieval	Output Format
DNA of Region	GCG FASTA NoHeader Decorated FASTA
Exon(s) only Sequence of selected gene/ORF (without introns)	GCG FASTA NoHeader

Supplementary Figure 3: Looking for homologous sequences in NCBI to find additional ITS regions in other species in addition to *C. albicans*.



Supplementary Figure 4: Aligning ITS regions in Geneious to help identify single base differences to design a primer.

The screenshot displays the Geneious (Restricted) 9.0.5 application window. The interface is divided into several panels:

- Top Panel:** Contains a toolbar with icons for Back, Forward, BLAST, Agents, Align/Assemble, Tree, and Primers (restricted). A search bar is also present.
- Left Panel (Sources):** A tree view showing the project structure, including Local (0), Sample Data, and Shared Databases. The 'Local (0)' folder is expanded, showing various sequence files.
- Center Panel:** Displays a list of sequences with columns for Name and Description. The 'Align/Assemble' view is selected, showing a sequence alignment. The alignment view includes a toolbar with icons for Extract, R.C., Translate, and other functions. The alignment shows two sequences, '1. Candida...' and '2. Candida...', with a consensus sequence below them. The consensus sequence is highlighted in green, and the alignment is shown in a color-coded format.
- Right Panel (Help):** A help panel titled 'Welcome to Geneious' with a 'Contents' link. It provides an introductory tour of the software's features and explains how to use the help panel.

The status bar at the bottom indicates 'g 137 / 996 MB men' and 'Features Restricted'.

Supplementary Figure 5: Designing a primer in geneious.

